IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE MATTER OF UNITED STATES PATENT APPLICATION NO: 10/814,244 ENTITLED "ANTI-RETROVIRAL ANALYSIS BY MASS SPECTROMETRY"

AND IN THE MATTER OF ESTABLISHING THAT (1) THE ARTICLE AUTHORED BY VOLOSEV ET AL., (CLINICAL BIOCHEMISTRY 2002 "SIMPLE RAPID METHOD FOR QUANTITATION OF ANTIRETROVIRALS BY LIQUID CHROMATOGRAPHY – TANDEM MASS -SPECTROMETER") IS THE INVENTOR'S OWN WORK and (2) THE INVENTOR IS THE SOLE INVENTOR.

DECLARATION OF STEVEN J. SOLDIN UNDER 37 CFR 1.132

Steven J. Soldin, being duly warned that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of the Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom, declares that:

1. I am a clinical biochemist/research scientist at Children's National Medical Center and Georgetown University. As such, I conduct medical research in many areas, one of which is the analysis anti-retroviral drugs.

2. I am the sole inventor of the subject matter described and claimed in the United States Patent Application Serial No. 10/814,244 filed April 1, 2004, entitled ANTI-RETROVIRAL ANALYSIS BY MASS SPECTROMETRY.

3. I assigned my rights to Children's National Medical Center in an assignment dated May 12, 2005 and recorded August 24, 2007 under reel/framed 019743/0038.

4. I am senior author of a paper in Clinical Biochemistry 2002 entitled "Simple rapid method for quantitation of antiretrovirals by liquid chromatography -- tandem mass - spectrometer", along with Andrew Volosov, Christopher Alexander and Lillian Ting, ("Volosov et al."; attached as Exhibit A) which was cited against the application in an office action dated January 2, 2008.

5. Andrew Volosov was a post doc student working under my direction and supervision and while co-authoring the publication, was not a co-inventor of the subject matter contained therein.

6. Christopher Alexander and Lillian Ting from the BC Centre for Excellence in HIV/AIDS, Vancouver, BC provided samples which we both measured, but were not involved in the development of the method, and while co-authoring the publication were not co-inventors of the subject matter contained therein.

7. As such, I am the sole inventor of the subject matter which is disclosed in Volosov et al. as published in Clinical Biochemistry and disclosed and claimed in United States Patent Application Serial No. 10/814,244 filed April 1, 2004, entitled ANTI-RETROVIRAL ANALYSIS BY MASS SPECTROMETRY.

8. This declaration is made in support of establishing that Volosov et al. is Applicant's own work, and is not made for any other or improper purpose.

9. The facts set forth in this Declaration are true, and all statements made of my own knowledge are true, and all statements made on information and belief are believed to be true.

Dated: 4-1-08

By: _____

Steven J. Soldin

EXHIBIT A

ELSEVIER

Clinical Biochemistry 35 (2002) 99–103

CLINICAL BIOCHEMISTRY

Simple rapid method for quantification of antiretrovirals by liquid chromatography—tandem mass-spectrometry

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Abstract

Objectives: The aim of the current study was to develop a simple, fast and universal method for quantification of any combination of all currently marketed anti-HIV drugs in human plasma, using a LC-tandem mass spectrometer (API-2000, SCIEX, Toronto, Canada).
Methods: 80 μL plasma were spiked with internal standard (cimetidine), and protein precipitated with 200 μL acetonitrile. The sample was centrifuged and 30 μL aliquot was injected onto the HPLC column, where it underwent an online extraction with ammonium acetate. After that the automatic switching valve was activated, changing the mobile phase to methanol and thereby eluting the analytes into the tandem mass spectrometer. Stavudine, AZT and efavirenz were analyzed in the negative MS/MS mode, while all other drugs were analyzed in the positive mode. The high selectivity of a tandem mass analyzer allows determination of any combination of the drugs within a 4.5 min run.
Results: Between-day precision was below 10% for all analytes at the concentrations tested. Accuracy ranged between 95% and 105% (n = 20). The method was linear over the measuring ranges of all analytes. Within-run precision gave a CV < 7% for all analytes. Good correlation with other analytical methods was observed.
Conclusions: The simplicity, universality and high throughput of the method make it suitable for application in a clinical laboratory. The method has been implemented in our laboratory for routine use.

Keywords: AIDS; HIV; Antiretrovirals; Chromatography; LC; MS; MS; Tandem mass-spectrometry; Anti-HIV

1. Introduction

Over the past several years, there has been a rapid increase in the number of marketed anti-HIV drugs. Currently there are fifteen marketed antiretrovirals (Table 1), with several more expected to reach the market in the near future. Anti-HIV treatment has become increasingly sophisticated and complex [1–3]. Cocktails of 3 to 4 drugs are used routinely in anti-HIV therapy. The specific choice of the drugs depends on a number of factors, such as drug resistance, tolerability, drug interactions and effectiveness of the treatment [4–6]. Combination of these factors (especially drug resistance) often leads to the need to alter the drugs prescribed to each patient. In such situations, therapeutic monitoring becomes an important factor.

The pharmacokinetics of many of the anti-HIV drugs are complex and sometimes unpredictable. Most of these drugs largely undergo oxidative CYP-3A4–mediated metabolism, which occurs primarily in the liver and gastrointestinal tract and is prone to numerous drug interactions and high inter- and intra-individual variability.

Although some of the anti-HIV drugs do not require TDM themselves, complicated regimens and drug cocktails with multiple drug interactions may justify TDM [7,8]. In addition, TDM may be the only way to effectively verify compliance, an issue which has been shown to be critical in HIV therapy [9]. The literature has shown that TDM of antiretrovirals is very useful for the protease inhibitors (PIs) and for the nonnucleoside reverse transcriptase inhibitors (NNRTIs) while its use for the nucleoside reverse transcriptase inhibitors (NRTIs) may be predominately to assess patient compliance [10,11,12].

A number of assays for simultaneous quantification of various groups of anti-HIV drugs (especially protease inhibitors) were reported in the literature [13–18]. However, to date no assay has covered the full spectrum of antiretro-

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Table 1
Marketed anti-HIV drugs

Protease Inhibitors (PI)	Nucleoside Reverse Transcriptase Inhibitors (NRTI)	Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)
Amprenavir	Abacavir	Delavirdine
Indinavir	Didanosine (ddI)	Efavirenz
Nelfinavir	Lamivudine (3TC)	Nevirapine
Ritonavir	Stavudine (d4T)	
Saquinavir	Zalcitabine (ddC)	
Lopinavir	Zidovudine (AZT)	

virals. In addition, a large difference in trough concentrations of different HIV drugs (see Table 2) represent a further challenge to development of a universal assay, requiring both the high sensitivity and ruggedness. The aim of the current study was to develop and implement an assay that would allow determination of any combination of all currently marketed anti-HIV drugs in a simple and fast procedure.

2. Materials and methods

2.1. Standards and chemicals

Standards of zalcitabine (ddC), didanosine (ddI) and zidovudine (AZT) were purchased from Sigma (St. Louis, MO, USA). Primary standards of efavirenz, indinavir, nelfinavir, nevirapine, ritonavir, saquinavir, lamivudine, abacavir and stavudine were obtained from the NIH Aids Reagent Reference Program (McKessonHBOC BioServices, Rockville, MD) while standards for amprenavir, delavirdine and lopinavir were isolated from commercially available tablets/capsules and characterized by H-NMR, UV spectroscopy and elemental analysis.

Methanol, acetonitrile, and ammonium acetate were purchased from Sigma and were of HPLC grade.

Table 2
Estimated limit of quantitation (LOQ) versus known trough concentrations of anti-HIV drugs*

Drug	Estimated LOQ (ng/mL)	Trough concentration* (ng/mL)
Efavirenz	1	—
Zidovudine (AZT)	3	<20
Stavudine (d4T)	3	20
Indinavir	<1	100
Abacavir	1	—
Nelfinavir	<1	1000
Delavirdine	<1	3000–8000
Saquinavir	<1	15–40
Nevirapine	1	3000
Lamivudine (3TC)	1	100–1000
Ritonavir	<1	1000
Amprenavir	<1	
Zalcitabine (ddC)	1	Undetectable
Didanosine (ddI)	1	100–300
Lopinavir	<1	—

2.2. Standard solutions and calibration curves

Stock solutions were prepared separately to obtain concentration of 0.1 mg/mL of each drug (total of 15 drugs). Methanol was used as a solvent.

Working Standard Solution was prepared by mixing equal amounts of stock solutions of each drug and diluting 1:4 with methanol to obtain a solution containing 1.67 μg/mL of each drug.

Seven-point calibration curve (blank and six calibrators) was prepared for calibration. Along with 50 μL of the internal standard solution 40 μL of working standard solutions were placed into 1.5 mL Eppendorf conical plastic test tubes, after which 40 μL of blank plasma were added to each test tube. The calibrators were further treated as described under Sample Preparation.

A solution of 0.15 mg/L of cimetidine in methanol was used as an internal standard (for both negative and positive MS/MS modes).

2.3. Sample preparation

For sample preparation, 80 μL of serum or heparinized plasma were placed into a 1.5 mL conical plastic Eppendorf test tubes containing 50 μL of internal standard solution and vortexed briefly. Then 200 μL of acetonitrile were added, the test tubes were capped, vortexed vigorously for 30 s and centrifuged at 14,000 g for 10 min. The supernatant was transferred into autosampler vials for injection into the LC-MS-MS system. Double-blank sample (sample that contains neither of the standards nor the internal standard) was also prepared with each calibration curve. Sample preparation was performed at room temperature. Plasma obtained from HIV-infected patients was heated for 30 min at 56°C to deactivate the HIV virus [19].

2.4. LC/MS/MS setup

An API-2000 tandem mass-spectrometer (SCIEX, Toronto, Canada) equipped with atmospheric pressure chemical ionization (APCI, heated nebulizer) source, two Perkin-Elmer PE-200 series micropumps and autosampler (Perkin-Elmer, Norwalk, CT, USA) was used to perform the analysis. Data processing was performed on Analyst 1.1 software package (SCIEX).

The main working parameters of the mass spectrometer are summarized in Table 3.

Table 3
Tandem mass-spectrometer main working parameters

Parameter	Value
Nebulizer temperature, °C	480
Dwell time per transition, msec	80
Nebulizer gas (Gas 1), psi	85
Auxiliary gas (Gas 2), psi	20
Curtain gas, psi	30
Nebulizer current, μA	2
Ion energy, V	0.8

2.5. LC/MS/MS procedure

The procedure is based on an online extraction/cleaning of the injected sample with subsequent introduction into the mass-spectrometer by using a built-in switching valve. 30 μL of the sample were injected onto Supelco LC-18-DB (3.3 mm x 3.0 mm, 3.0 μm ID) chromatographic column equipped with Supelco Discovery C-18 (3.0 mm) guard column (Supelco, St. Louis, MO, USA). The sample underwent cleaning with an aqueous solution of ammonium acetate (15 mM) at a rate of 1 mL/min. After 2.4 min of cleaning the switching valve was activated, the column was flushed with methanol at a rate of 1 mL/min and the sample was introduced into the mass-spectrometer. Analytes were then quantified by multiple reaction monitoring (MRM) (see Table 4 for MRM transitions). MRM allows for enhanced selectivity through the measurement of parent and daughter ions simultaneously for each of the compounds of interest. Due to a high selectivity of a tandem mass analyzer, no chromatographic separation was necessary for quantification of the analytes and the analysis was complete less than a minute after activation of the switching valve. Total analysis time was 4.5 min, including equilibration time before the next injection. The procedure was completely automatic and controlled by the Analyst 1.1 software.

Table 4
MS/MS ion transitions and modes of analysis

Drug	Ionization mode	MS/MS transition
Efavirenz	Negative	314.2/69.0
Zidovudine (AZT)	Negative	266.2/222.8
Stavudine (d4T)	Negative	223.0/42.0
Indinavir	Positive	614.5/421.3
Abacavir	Positive	287.1/191.1
Nelfinavir	Positive	568.3/330.1
Delavirdine	Positive	457.2/221.1
Saquinavir	Positive	671.4/570.3
Nevirapine	Positive	267.2/226.1
Lamivudine (3TC)	Positive	230.1/112.0
Ritonavir	Positive	721.3/268.2
Amprenavir	Positive	506.3/245.2
Zalcitabine (ddC)	Positive	212.1/112.0
Didanosine (ddI)	Positive	237.1/137.1
Lopinavir	Positive	629.3/447.3

2.6. Drug interference studies

The following commonly used drugs were tested at both their therapeutic and toxic concentrations for potential interference in the procedure described: acetaminophen, amikacin, caffeine, carbamazepine, digoxin, disopyramide, ethosuximide, flecainide, gentamicin, lidocaine, lithium, methotrexate, N-acetylprocainamide, phenobarbital, phenytoin, primidone, procainamide, quinidine, salicylate, theophylline, tobramycin, valproic acid, and vancomycin.

2.7. Drug comparison studies

Comparison studies were performed for the PIs and NNRTIs using the tandem MS/MS method utilized at the BC Center for Excellence in HIV/AIDS.

2.8. Quality control and proficiency testing

Serum or plasma spiked with known concentrations of the drugs and at 3 levels (low, medium and high) were used as daily quality controls. External proficiency testing is available from "International Quality Control Program for Therapeutic Drug Monitoring in HIV Infection" (University Medical Center Nijmegan, Department of Clinical Pharmacy, PO Box 9101, 6500 HB Nijmegan, The Netherlands)

3. Results and discussion

Accuracy and precision were evaluated by analyzing quality control samples at low, medium and high concentrations on 20 different days. Within-run precision (%CV) was below 7% for all analytes. Between-day precision (%CV) was below 10% for all analytes at the tested concentrations. Accuracy (% of weighed-in target concentration measured) ranged between 95% and 105%. The results are summarized in the Table 5. The assay was linear over the range of 2 to 2000 ng/mL for stavudine, didanosine, zalcitabine and AZT, and 10 to 10000 ng/mL for all other drugs.

None of the drugs listed above interfered in the method described.

The performance of the method was also compared with other analytical methods used for quantitation of nine of the tested drugs. A total of ~600 clinical samples containing various combinations of amprenavir, indinavir, saquinavir, delavirdine, efavirenz, lopinavir, ritonavir nelfinavir, and nevirapine were analyzed in our lab by the new method, as well as by using a different LC-MS-MS method used routinely for the TDM of HIV drugs in British Columbia. The study was performed in a blinded manner and the laboratories did not share the results until after the completion of the study. Specimens were transported on dry ice and sent by overnight courier. They were stored at −70 C until analyzed. These drugs are known to be stable at 4 C for 1

Table 5
Inter-day accuracy and precision data

	n	Mean (%)			% CV		
		10 ng/mL	100 ng/mL	500 ng/mL	10 ng/mL	100 ng/mL	500 ng/mL
Efavirenz	20	104.5	102.6	101.4	9.9	8.7	6.2
Zidovudine (AZT)	20	95.0	96.1	103.2	9.5	9.3	5.1
Stavudine (d4T)	20	104.7	101.5	102.6	8.9	7.9	8.0
Indinavir	20	104.1	98.2	101.0	7.6	6.0	3.2
Abacavir	20	96.8	97.9	97.5	8.7	6.2	5.5
Nelfinavir	20	95.9	104.3	102.0	8.4	6.3	4.2
Delavirdine	20	104.3	96.9	99.8	8.2	5.2	6.0
Saquinavir	20	103.9	101.8	98.7	9.5	7.1	4.1
Nevirapine	20	95.5	103.1	101.9	6.9	4.2	5.9
Lamivudine (3TC)	20	98.7	103.0	100.8	8.8	4.9	2.1
Ritonavir	20	104.1	98.2	97.5	7.6	6.3	3.9
Amprenavir	20	103.5	99.4	99.0	8.6	7.4	4.5
Zalcitabine (ddC)	20	96.4	101.9	103.0	9.8	7.1	3.9
Didanosine (ddI)	20	97.7	103.6	100.1	9.7	6.1	6.5
Lopinavir	20	95.0	103.9	102.9	5.2	1.8	1.8

week. The results are summarized in the Table 6. As can be seen from the Table 6, the results correlated very well for all of the tested drugs. Differences in the regression slopes can be explained by the differences in standardization between the two laboratories. Due to the difficulty in obtaining standards from the drug companies [14], both of the laboratories purified the drugs initially from tablets. Gold standards for these drugs were only recently obtained by our laboratory from the NIH and were used to recalibrate our standards, perhaps accounting for some of the differences between the laboratories. Specimens for correlation studies of the NRTIs were unavailable. The NRTIs are active intracellularly as their triphosphates. Other than to assess compliance TDM has not been found to be useful for this group of drugs.

As was discussed elsewhere [20], the use of the APCI source minimized the potential for ionization suppression and matrix effects. Co-elution of the analytes and the internal standard further helped to overcome these problems, also eliminating the need to use internal standards structurally related to the analytes, which would be impossible considering the number of monitored drugs. Cimetidine was chosen as internal standard as it was suitable for both ionization modes, further simplifying the procedure.

If the combination of the drugs taken by the AIDS patient is not known, monitoring of all fifteen drugs may be needed. This would result in a simultaneous monitoring of thirteen ion transitions in the positive mode. In such a case care should be taken to adjust dwell times and delays between ion transitions so that there are at least ten to twelve data points for each monitored peak. Failure to meet this requirement may result in inadequate peak integration and considerably worse precision. This, however, can occur only in the most complicated scenario, when the analyst does not know which anti-HIV drugs were taken by the patient, which is rarely the case in clinical practice.

In conclusion, the method we developed is easy and reliable and allows for the simultaneous measurement of any combination of 15 AIDS drugs in less than 5 min thereby facilitating TDM for both the PIs and the NNRTIs.

Table 6
Linear regression parameters calculated from method comparisons (see text)

	n	Slope	Y-intercept	X-intercept	r	$S_{y.x}$
Amprenavir	16	0.54 ± 0.06	270.8 ± 146.1	−498.7	0.911	304.0
Delavirdine	10	0.79 ± 0.03	266.6 ± 231.4	−338.1	0.993	425.9
Nevirapine	28	0.78 ± 0.07	506.5 ± 230.3	−546.0	0.928	428.5
Lopinavir	32	0.87 ± 0.05	558.0 ± 381.1	−642.9	0.949	944.2
Efavirenz	15	1.15 ± 0.08	150.8 ± 188.8	−131.5	0.971	398.4
Ritonavir	52	0.96 ± 0.02	−4.8 ± 44.4	5.05	0.992	266.7
Saquinavir	26	0.91 ± 0.03	11.0 ± 18.4	12.0	0.989	71.08
Nelfinavir	33	0.73 ± 0.02	88.5 ± 41.5	−121.7	0.988	146.8
Indinavir	98	1.16 ± 0.02	−77.1 ± 34.4	66.6	0.983	252.2

$S_{y.x}$ = standard deviation of the residuals.

Acknowledgment

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Author Keywords: AIDS; HIV; Antiretrovirals; Chromatography; LC; MS; MS; Tandem mass-spectrometry; Anti-HIV

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